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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF

KISIELOW ET AL.

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FOR: METHODS OF OBTAINING ISOFORM SPECIFIC EXPRESSION IN MAMMALIAN CELLS

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DECLARATION BY MALGORZATA ANNA KISIELOW PURSUANT TO 37 C.F.R. §1.131

I, Malgorzata Anna Kisielow, a citizen of Poland, hereby declare as follows:

1. I am the inventor of the subject matter described and claimed in the above-identified patent application.

2. Prior to November 2001, I had completed my invention as described and claimed in the above-identified patent application in Switzerland, a WTO country, as evidenced by the following:

Prior to November 2001, I conceived and tested the method of isoform-specific siRNA knockdown described and claimed in the above-identified application as evidenced by the poster from the 2001 annual meeting of the Friedrich Miescher Institute for Biomedical Research attached hereto as Exhibit A, this meeting took place prior to Novemebr 2001;

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

M. Kisielow — Jan 15, 2007

Malgorzata Anna Kisielow — Date

154 Isoform-Specific Knock-Down and Knock-In In a Week

Malgorzata Kisielow, Michiaki Nagasawa & Yoshikuni Nagamine

Introduction

RNA interference (RNAi) is a process in which double-stranded RNA molecules turn off, or silence, the expression of a gene with a corresponding sequence. The molecular mechanism of RNAi is not completely understood, but it is known that short interfering RNAs, double stranded fragments 21-23 bps in length, are the mediators of this highly specific process (see Fig1). Recently, it was reported that the introduction of chemically synthesized siRNAs can suppress gene expression in mammalian cells. We used this approach to knock-down ShcA in HeLa cells. ShcA is an adapter molecule involved in many processes including mitogenesis, transformation, apoptosis and cytokine production. It exists in three isoforms p66, p52 and p46 that differ only in their amino-terminal regions (see Fig2). We also tried to silence and express ShcA in an isoform-specific manner.

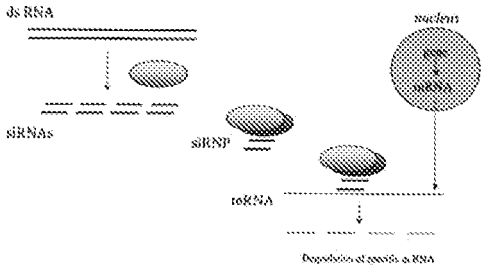

Fig.1 RNA interference scheme. dsRNA is processed to 21-23mers (short interfering RNAs or siRNAs) by RNaseIII type protein (green oval). siRNAs guide the nuclease complexes (siRNP- a small interfering ribonucleoprotein /RISC-RNA induced silencing complex) to the target sequence, which results in degradation of target m RNA.

Results

By introducing siRNAs designed against the sequence common to all three isoforms (Fig. 3A) we managed to reduce ShcA expression by over 95% in HeLa cells (Fig. 3B), and this effect was stable for 5-6 days (Fig. 3C).

siRNAs specific for p66 isoform (Fig. 4A) suppressed the expression of p66ShcA and did not affect p46 and p52 isoforms (Fig. 4B), this effect was also stable for 5-6 days (Fig. 4C).

To get isoform-specific expression , all three isoforms were silenced with a human specific siRNA followed by the introduction of expression vectors encoding mouse ShcA isoforms. This indeed resulted in the expression of individual p46, p52 or p66 isoform (Fig. 5).

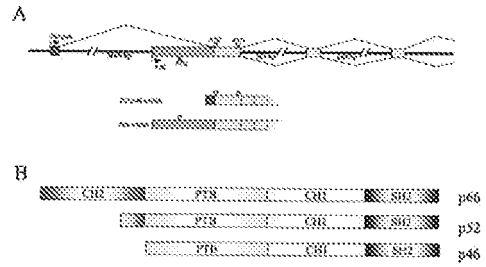

Fig.2 ShcA exists in three isoforms: p66 resulting from differential transcription initiation, p52 and p46 resulting from differential translation initiation.
A- partial ShcA gene structure; 1,2,3- exons, triangles-translation start sites,
B- ShcA proteins; PTB-phosphotyrosine binding domain, SH2-Src homology 2 domain, CH1,CH2-proline/glycine rich regions.

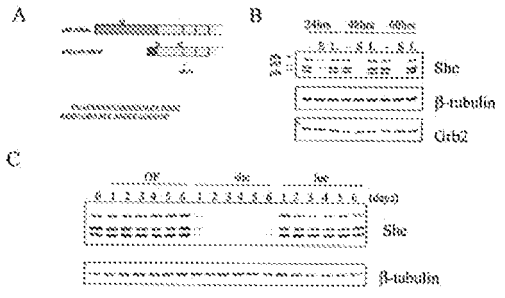

Fig.3 Knock-down of ShcA in HeLa cells. A- the location and sequence of ShcA specific siRNAs (Sshc), B- knock-down of all three isoforms , C- time course, L/luc- non-specific siRNAs control, - untreated cells.

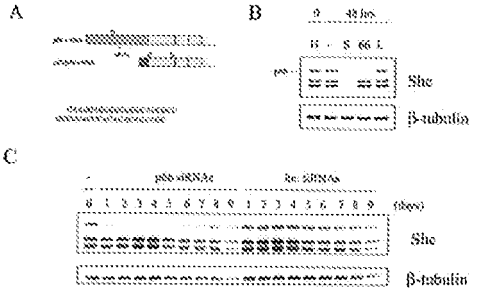

Fig.4 Isoform-specific knock-down of ShcA in Hela cells. A- the location and sequence of p66ShcA specific siRNAs . B- knock-down of all three isoforms (S), knock-down of p66 isoform (66), M- untreated cells, - transfection reagent control, L/luc - non-specific siRNAs control, C-time course.

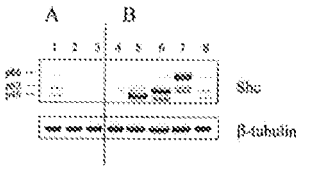

Fig.5 Knock-down of ShcA with human specific siRNAs and knock-in of mouse ShcA isoform(S) in HeLa cells. A- knock-down of ShcA. *Lane* 1, untreated; *lane* 2, siRNA common to human and mouse ShcA; *lane* 3, siRNA specific to human ShcA. B - 24 hrs after treatment with human-specific ShcA siRNA, cells were transfected with Lipofectamine 2000 alone (lane 4), or expression vectors for p46ShcA (lane 5), p52ShcA (lane 6), and p66ShcA (lane 7). Lane 8, untreated HeLa cells. Samples were collected 48 hours post-siRNAs-transfection.

Discussion

Many eukaryotic genes are expressed in multiple isoforms. For the study of individual isoforms in a clear background, a conventional approach is to ectopically express the individual wild-type or mutant isoform in cells or animals in which the target gene is deleted, a lengthy procedure that can take up to several months.

Using a new and efficient method for gene silencing, siRNAs, we show that a particular isoform of ShcA protein can be silenced and/or expressed in mammalian cells in less then a week. The silencing effect remains stable for at least 5 days, which provides a sufficient window of time in which to conduct experiments.

Now, we will use this approach to study the effect of different ShcA isoforms on growth factor-induced signaling.